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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Koichi MASUDA *et al.*

Serial No.: 10/084,640

Filed: February 25, 2002

Title: ENGINEERED INTERVERTEBRAL DISC TISSUE

Docket No.: 047940-0139

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TECH CENTER 1600/2900

Commissioner for Patents
Washington, D.C. 20231

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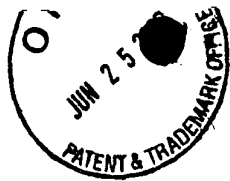
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Atty. Dkt. No. 047940-0139

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Applicant: Koichi MASUDA *et al.*
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Appl. No.: 10/084,640
Filing Date: 02/25/2002
Examiner:
Art Unit: 1651

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CLAIM FOR PRIORITY UNDER 1.78(a)(2)

Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants hereby claim priority to U.S. Patent Application No. 60/462,513, filed November 9, 2001, entitled "Osteogenic proteins, including BMPs and CDMPs, and their role in musculoskeletal tissue development and repair, including bone and non-mineralized tissues" (Rueger), a copy of which is enclosed herewith. Applicants believe no fee is due for this submission, however, the Commissioner is hereby authorized to charge any fees due in connection with this submission to Deposit Account No. 06-1447. A duplicate copy of this Claim is enclosed for such purposes.

REMARKS

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Respectfully submitted,

Date June 25, 2002

By 

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PROVISIONAL
APPLICATION FOR
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Attorney Docket
No.

STK-07-02

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First Named
Inventor

David C. Rueger

JUL 01 2002

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

INVENTOR(S)

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Family Name or Surname

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Residence

(City and either State or Foreign Country)

Southborough, Massachusetts

TITLE OF THE INVENTION (280 characters max)

Osteogenic proteins, including BMPs and CDMs, and their role in musculoskeletal tissue development and repair, including bone and non-mineralized tissues

ENCLOSED APPLICATION PARTS (check all that apply)

- | | |
|--|--|
| <input checked="" type="checkbox"/> Specification (Number of Pages 36) | <input type="checkbox"/> CD(s) (Number) |
| <input type="checkbox"/> Drawing(s) (Number of Sheets) | <input checked="" type="checkbox"/> Application Data Sheet |
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

- ☐ Applicant claims small entity status.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Application Information

Title Line One:: Osteogenic proteins, including BMPs and CDMPs, and their
Title Line Two:: role in musculoskeletal tissue development and repair,
including bone and non-mineralized tissues
Total Drawing Sheets:: 0
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Application Type:: Provisional
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Licensed US Govt. Agency::
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Representative Information

Representative Customer Number:: 021323

Continuity Information

This application is a::
>Application One::
Filing Date::
Patent Number::
which is a::
>>Application Two::
Filing Date::
Patent Number::

Prior Foreign Applications

Foreign Application One::
Filing Date::
Country::
Priority Claimed::

UNITED STATES PROVISIONAL PATENT APPLICATION

of

David C. Rueger

for

**OSTEOGENIC PROTEINS, INCLUDING BMPS AND CDMPS, AND THEIR ROLE IN
MUSCULOSKELETAL TISSUE DEVELOPMENT AND REPAIR, INCLUDING BONE
AND NON-MINERALIZED TISSUES**

AGE-RELATED CHANGES IN ENDOGENOUS OSTEOGENIC PROTEIN-1 IN HUMAN KNEE ARTICULAR CARTILAGE

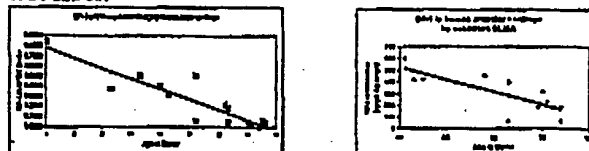
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INTRODUCTION. As progressive cartilage degeneration seen in osteoarthritis (OA) increases markedly with age, it is important to learn more about potential anabolic factors aimed to prevent or protect cartilage from degradation and/or promote its repair. It is especially important since there is a decline in the ability of chondrocytes to repair with age. As we recently documented (1), human newborn and adult articular chondrocytes endogenously produce one of these factors, osteogenic protein-1 (OP-1), a "repair" factor that has a significant anabolic effect on human and bovine adult articular cartilage. OP-1 induces synthesis of major matrix components, counteracts the catabolic effect of interleukin-1 and fibronectin fragments, promotes cartilage matrix assembly by an up-regulation of CD44 and hyaluronan synthase-2, but has little effect on chondrocyte proliferation. Importantly, our previous data indicate two forms of OP-1 protein in human articular cartilage: an inactive (unprocessed, pro-) form and an active (processed, mature) form. However, only mature, active form has been shown to induce the anabolic effect. The hypothesis has been developed that with aging human articular cartilage shows evidence of a decrease in endogenous OP-1 content, synthesis and metabolism thus leading to an elevated susceptibility of cells to catabolic processes and contributing/promoting cartilage degeneration. In order to test this hypothesis the objective of the current study was to use quantitative approaches to estimate the levels of message and protein of endogenous OP-1 in human adult articular cartilage from donors of different age.

MATERIALS AND METHODS. Full thickness articular cartilage was dissected from 20 human adult donors with no documented history of joint disease through the collaboration with the Regional Organ Bank of Illinois. Tissue was obtained from the load-bearing region of the femoral condyle. Three methods were applied for the quantification of the levels of message and protein of endogenous OP-1 in these samples. OP-1 mRNA expression was measured by using nested RT-PCR method based on the direct extraction of total RNA from tissue. Densities of the PCR bands were evaluated using a Fluor-S Multimager with attached software program and were normalized to the densities of the GAPDH. OP-1 primers utilized in this study were described previously (1). Content of total OP-1 protein was estimated by a newly developed sandwich ELISA and by western blotting. For sandwich ELISA tissue was lyophilized and OP-1 protein was extracted with Lysis buffer, pH 7.5. Human recombinant mature OP-1 and two antibodies, one monoclonal antibody against the entire mature domain of OP-1 and one polyclonal antibody against the synthetic peptide of 18 amino acid close to the N-terminus end, were used for this assay. ELISA results were normalized to the dry weight of the tissue. The same antibodies were applied for western blotting. The densities of specific immunoreactive bands were analyzed with Fluor-S Multimager. Western blot results were normalized to the total protein content.

RESULTS. For these studies on aging only cartilage from normal organ donors with no documented history of joint disease was used. Aliquots of tissue were used for mRNA and protein extraction. The results of semi-quantitative RT-PCR (left graph) indicated the highest levels of mRNA expression in newborn and young adult donors, while with aging this expression was downregulated. When OP-1/GAPDH ratio was plotted onto the graph, results indicated a clear decline in the levels of OP-1 mRNA expression with age. The highest levels of OP-1 message were detected in newborn cartilage. In adult tissues the changes in OP-1 expression had a linear regression and by age of 80 OP-1 message was very low or barely detectable,

some donors at old age had the levels of expression even below the detection limit. There were at least 4-5-fold differences in message levels between age of 30 and 80.



Similar results were obtained with quantitative ELISA method (right graph). For the first time we were able to quantify the absolute content of total endogenous OP-1 in human adult articular cartilage in ng quantities by using a method developed by us. We have tested the variety of extraction protocols, the effect of pH and the effect of extractants on the ELISA sensitivity and accuracy. The optimal conditions were selected and by this ELISA method we found that the content of total OP-1 in human normal adult articular cartilage at the age of 40 is between 400 to 600 ng/g of dry tissue. ELISA results supported PCR data and confirmed the linear decrease in the content of endogenous OP-1 protein as well. A 3-4 fold decrease was observed in a comparison between donors that were over 70 years old to donors around 40. When the same cartilage extracts were analyzed by western blotting using separate antibodies to pro- and mature forms of OP-1, similar pattern in changes of endogenous OP-1 protein was found. Moreover and very important, major differences were noticed on the level of the cleaved mature OP-1. With age the amount of active mature OP-1 was significantly decreased.

DISCUSSION. These studies for the first time report the changes in endogenous OP-1 that occur in human adult articular cartilage with aging on both levels, mRNA and protein. Critically, new quantitative approaches were developed in order to monitor these alterations. As we show, the concentration of endogenous OP-1 protein detected by our ELISA method in human adult articular cartilage is within 100-600 ng/g of dry weight. This content is dramatically decreased with aging when compared to young adults. Interestingly, variations in endogenous OP-1 occur parallel in mRNA and protein levels. Our data indicate that with age there is not only a decrease in total OP-1 available for the anabolic response of chondrocytes, but what is more important, there is a decrease in processed, mature OP-1. Our results indicate a strong correlation between the levels of endogenous OP-1 and the age of human donors supporting the hypothesis that with aging articular cartilage displays a decrease in endogenous anabolic factors, that could contribute to higher susceptibility of articular cartilage to degenerative processes such as seen in OA. The reported ELISA method could be developed as a valuable diagnostic or prognostic tool for predicting the possibility of pathophysiological changes in connective tissues.

REFERENCES. 1) Chubinskaya et al. (2000) J Histochem & Cytochem 48:239-250.

ACKNOWLEDGMENTS. Collaboration with Allan Valdelion MD of the Regional Organ Bank of Illinois and his staff is gratefully acknowledged. The authors thank Arcady Margulis, MD, for procurement of human donor cartilage, Gabriela Ca-Szabo, Ph.D. and Richard Berger, MD, for providing OA cartilage.

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BMP RECEPTORS SPECIFIC FOR OP-1 ARE IDENTIFIED IN HUMAN NORMAL, DEGENERATIVE AND OA CARTILAGE

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INTRODUCTION. Osteogenic protein-1 (OP-1) is a member of bone morphogenetic protein (BMP) family that has been shown to have an important function in stimulating cartilage homeostasis when applied exogenously. Our recent data (1) demonstrated that human articular chondrocytes from normal and osteoarthritic (OA) cartilage express OP-1 endogenously thus indicating the potential for cartilage to repair. OP-1 transduces signals through *trans*-membrane serine-threonine kinase receptors known as type I and type II receptors (BMPR-II). OP-1 shares these receptors with other BMP molecules. Both type I and type II receptors belong to large families of proteins. Among them at least three type I receptors have been characterized as OP-1 receptors: activin-receptor-like kinase 2 (ALK2), BMP receptor-1A (BMPR-1A, or ALK3) and BMPR-1B (or ALK6) (2). The detection of endogenous OP-1 in cartilage and the anabolic effect shown by exogenous OP-1 suggest the possibility for the OP-1 signaling pathway to be actively present in articular cartilage. Critical questions relevant to our understanding of OP-1 function in articular cartilage are: 1) which receptors are expressed in articular cartilage; 2) which receptor combination occurs *in vivo* in cartilage under normal and pathological conditions; 3) whether different type I receptors trigger specific downstream pathways. Previously we reported the presence of OP-1 receptors in rabbit articular cartilage (3). The purpose of the current study was to identify the OP-1 receptors present in human articular cartilage and the differences in receptor types, levels of mRNA and protein expression and their distribution between normal and OA cartilage.

MATERIALS AND METHODS. Full thickness articular cartilage was dissected from load bearing regions of the femoral condyle of donors with no history of joint disease within 24 hours of death. After opening the joint, the surface of the cartilage was grossly examined. The appearance of each joint was given a grade based on the Collins scale modified by Muehleman et al (4). Cartilages with the grade II and higher were called degenerative. Human OA cartilage was obtained from patients who have undergone knee arthroplasty due to advanced OA. Total RNA was extracted directly from tissue. Specific primer pairs and optimal PCR conditions were designed for each receptor type. Densities of the PCR bands were measured using a Fluor-S MultiImager with attached software program and were normalized to the densities of the GAPDH. For each receptor type specific polyclonal antibodies were used to study the tissue distributions (by immunohistochemistry), cellular distribution (by immunofluorescence) and protein size (by western blotting). For immunohistochemistry, cartilage was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (6µm). For immunofluorescence, chondrocytes were released from the tissue with 0.2% pronase in DMEM containing 5% FBS for 1.5 hours, incubated overnight with 0.025% collagenase P and plated on dual chamber slides in monolayer at high-density. The culture was maintained for 2 days in DMEM to allow the cells to recover. After culture, cells were fixed in 4% paraformaldehyde. Primary antibodies were applied followed by application of fluorescently labeled secondary antibody. The localization of OP-1 receptors was viewed by fluorescent microscope. Proteins were extracted from cartilage with 1 M GuHCl buffer or separated with sucrose gradient as membrane-enriched or soluble fractions (5) and were analyzed by western blotting with the same antibodies.

RESULTS. In human articular cartilage we have identified mRNA and

protein expression for type I and II OP-1 receptors. By using antibodies against these receptors (BMPR-II, ALK-2, ALK-3 and ALK-6) primarily cell-associated specific immunostaining was found in normal and OA cartilage. The strongest cellular staining in normal cartilage was found in the superficial and upper middle layer chondrocytes, while in OA cartilage cells from the deep layer were also stained positively. Some light matrix staining was detected for type I receptor (more for ALK-2 than for ALK-3) in the superficial and upper middle layer of normal cartilage. The intensity of the interterritorial matrix staining and its distribution were increased in the degenerative and OA cartilage. Similar pattern of immunostaining was observed for BMPR-II. This type II receptor was mostly co-localized with type I receptor. However, in addition, BMPR-II was found also in the territorial matrix of degenerative and OA cartilage. By western blot analysis OP-1 receptors were detected in total cartilage extracts as well as in membrane-enriched fraction. The intensity of immunoreactive bands for type I receptors was higher in OA samples when compared to normal, while the intensity of BMPR-II bands was stronger in normal tissue than in OA. Interestingly, type I OP-1 receptors were present in OA cartilage also in the soluble fraction. In normal adult cartilage by immunofluorescence all receptors were visualized on the membranes of isolated chondrocytes. Comparative analysis of mRNA levels for each receptor type showed that BMPR-II, ALK-2 and ALK-3 message was elevated two-three fold in degenerative tissues (grade II and III) and in OA cartilage. Expression of ALK-6 did not show substantial changes, although some decrease in message was noticed.

DISCUSSION. In the current study we report for the first time that all known OP-1 receptors are expressed by human adult normal and OA chondrocytes. We identified both message and the protein for these receptors. As comparative analyses show the levels of mRNA and protein expression as well as protein distribution correlates with the pathophysiological state of the tissue. The strongest staining for OP-1 receptors was found in the upper part of the cartilage indicating their co-localization with the ligand (matric OP-1). The matrix staining for the receptors in the degenerative and OA cartilage and their appearance in the soluble fraction suggest a possible shedding from the cell membrane during process of cartilage degeneration. Importantly, the parallel changes in the message levels for BMPR-II and only for two out of three type I receptors (ALK-2 and ALK-3) indicate, perhaps, the primary involvement of these receptors in OP-1 signaling pathway in human degenerative and OA cartilage. In conclusion, our study confirms that not only the ligand (OP-1) but also its receptors depend on the pathophysiological state of the tissue. This study provides a critical background for further understanding of the role of OP-1 and OP-1 signaling in cartilage homeostasis under physiological and pathophysiological conditions.

REFERENCES. 1) Chubinskaya et al (2000) J Histochem Cytochem 48:239-250. 2) Ten Dijke et al (1994) JBC 269:16985-16988. 3) Muehleman et al (1999) Trans ORS 46(2):1050. 4) Muehleman et al (1997) Osteoarthritis & cartilage 5:23-37. 5) Böhm et al (1994) Arch Biochem Biophys 314:64-74.

ACKNOWLEDGMENTS. Collaborations with Allen Veldellon, MD, of the Regional Organ Bank of Illinois, Aready Margulis, MD, Gabriella Ca-Szabo, Ph.D., and Richard Berger, MD, are acknowledged.

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FORMATION OF TRANSPLANTABLE DISC-SHAPED TISSUES BY NUCLEUS PULPOSUS AND ANULUS FIBROSUS CELLS: BIOCHEMICAL AND BIOMECHANICAL PROPERTIES

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[INTRODUCTION] Degenerative intervertebral disc (IVD) disease is associated with a decrease in the contents of matrix proteoglycans and collagens. Few attempts have been made to repair damaged IVD by transplanting a matrix-rich tissue formed *in vitro* into the defect. Recently, we have developed a novel two-step culture method using alginate-recovered-chondrocyte (ARC) for the production of cartilaginous tissue *in vitro*; this method alleviates the need for using exogenous matrices [1]. The purpose of this study was to test the possibility of combining these approaches to form *in vitro* a cohesive tissue for transplantation *in vivo*.

[MATERIALS AND METHODS]

Cell Isolation and ARC Culture Methods Bovine intervertebral disc cells from the tails of 14-18 month old steers were isolated by sequential enzyme digestion [2]. The ARC Method was then used as follows to form discs *in vitro*. Annulus fibrosus (AF) cells and nucleus pulposus (NP) cells were separately cultured in beads of 1.2% low viscosity alginate (Kelton LV, Kelco) at 4 million cells/ml using daily changes of DMEM/F12 medium containing 20% FBS + OP-1 (200 ng/ml), 25 µg/ml ascorbate and 10 µg/ml gentamicin. The cells with their cell-associated matrix were recovered by centrifugation of alginate beads solubilized in the presence of sodium citrate after 10 days of culture in alginate. The pelleted cells were resuspended in complete medium containing 20% FBS and 200 ng/ml of OP-1, seeded onto a tissue culture insert with a porous membrane (Transwell; 0.4 µm pore size; Costar; 10 mm diameter) and maintained in daily changes of the same medium for up to 4 weeks.

Characterization of Engineered Tissues *in vitro* After 2 and 4 weeks, the *de novo* formed tissue was separated in each case from the porous membrane; the weights (dry and wet) were measured and the tissue was subjected to biochemical analyses. Each tissue was also examined histologically. The contents of sulfated PG and DNA were measured by the DMMB method and a Hoechst 33258-dye method, respectively. The contents of collagen and pyridinoline were measured by reverse-phase high-performance liquid chromatography. Compressive and tensile testing were performed to determine the equilibrium compressive modulus, H_{eq} , the hydraulic permeability at 15% of strain, k_{15} , and the peak tensile stress σ_{max} . The data were analyzed statistically using ANOVA.

[RESULTS] After 2 weeks, tissues engineered from NP and AF cells had a disk-like structure and were easy to separate from the membrane (Figure 1). The presence of OP-1, at a concentration of 200 ng/ml, stimulated the formation of cohesive discs. Interestingly, both the wet and dry weights and also the thickness of the NP discs were significantly higher than those of AF discs (Table 1). The water contents of NP discs were also significantly higher than those of AF discs (Table 1). Significant proteoglycan accumulation was observed in both NP and AF discs, but especially in the former ($p < 0.01$) (Figure 2A). On the other hand, the collagen content of the AF discs was greater than that of NP discs ($p < 0.01$) (Figure 2B). H_{eq} and σ_{max} varied significantly with cell types (each $p < 0.01$) but not culture duration ($p = 0.47$ and 0.17 , respectively). H_{eq} and σ_{max} of AF tissue were significantly higher (+170% and +270%, respectively) than those of NP tissue (Table 1). k_{15} was lower for AF discs than NP discs ($p < 0.05$), and increased with culture duration ($p < 0.05$), without an interactive effect ($p = 0.75$).

[CONCLUSION] The results show that the recently described ARC method used to form cartilaginous tissue *in vitro* can also be used to form a disc-shaped tissue by IVD cells. Importantly, the collagen content was higher and the ratio of proteoglycan/collagen was lower in the AF than in the NP tissue,

consistent with the observation that AF cells form a more fibrous tissue than NP cells *in vitro* and with the observed different mechanical properties of the engineered tissues using AF and NP cells. The results obtained thus far suggest that IVD tissues may be engineered *in vitro* using different cell sources (AF and NP) and that this process can be stimulated by growth factors such as OP-1. It remains to be determined if such tissues can be transplanted *in vivo* to repair IVD defects and/or degeneration.

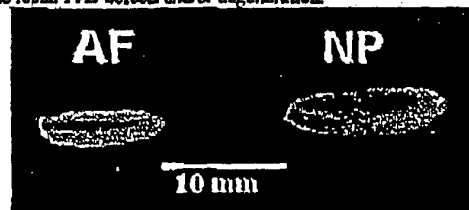


Figure 1. Gross Appearance of Tissues (2 weeks).

Table 1. Characterization of Engineered Tissues

	Culture Duration	AF disc	NP disc
Wet Weight (mg/tissue)	2 w	49.4 ± 1.7	132.7 ± 14.1
	4 w	159.4 ± 6.0	**166.9 ± 5.2*
Dry Weight (mg/tissue)	2 w	3.08 ± 0.09	7.21 ± 0.86*
	4 w	5.52 ± 0.09	7.34 ± 0.25***
Water Content (%)	2 w	93.8 ± 0.3	95.8 ± 0.1***
	4 w	94.1 ± 0.1	95.6 ± 0.1***
Thickness (mm)	2 w	0.49 ± 0.13	1.37 ± 0.19***
	4 w	0.60 ± 0.17	1.11 ± 0.57***
H_{eq} (kPa)	2 and 4 w	2.44 ± 0.30	0.92 ± 0.16**
σ_{max} (kPa)	2 and 4 w	250 ± 37	87 ± 13***
$\log(k_{15})$	2 w	-13.3 ± 0.2	-13.6 ± 0.3
	4 w	-13.0 ± 0.3	-12.4 ± 0.4

(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ versus AF)

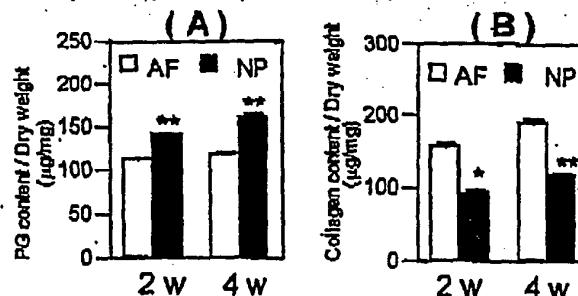


Figure 2. PG (A) and Collagen (B) Content of Engineered AF and NP Tissues (* $p < 0.05$, ** $p < 0.01$ versus AF)

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A NOVEL CULTURE SYSTEM FOR THE INTERVERTEBRAL DISC: AN ORGAN CULTURE SYSTEM WITH ENDPLATES

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INTRODUCTION:

The intervertebral disc (IVD) consists of two different tissues, the nucleus pulposus (NP) and annulus fibrosus (AF). These tissues are integrated to allow the spinal segments to articulate and bear load. Nutrients for the cellular elements of the NP and AF are transported through adjacent endplates located in the caudal and cranial sides (see Figure 1).

The pathogenesis of IVD degeneration is poorly understood. Recent studies have led to the development of new culture methods that have proved useful in shedding light on the metabolism of IVD cells and tissues [1, 2]. However, there has been, as far as we know, no attempt to include the endplates as part of the IVD unit cultured.

Osteogenic protein-1 (OP-1) administered *in vivo* to the IVD has positive effects on the metabolism of the injected spinal tissues [3]. The goals of the current study were: (1) to establish a new method to culture a disc with endplates; and (2) to investigate the usefulness of this model in studying the effects of an intradiscal OP-1 injection.

MATERIALS AND METHODS:

Tissue Culture Model:

Lumbar spines were removed *en bloc* under sterile conditions from New Zealand White rabbits weighing 3.5-4.0kg, immediately after the rabbits were euthanized. IVDs containing end plates were dissected from consecutive levels (L1-L6) (see histology, Figure 1). After three rinses with physiologic saline, each IVD was placed in an individual well of a 12-well culture plate. The discs were assigned to one of three groups. Discs in the first group (NT Group) were cultured for an additional 14 days in complete medium [DMEM/F-12 medium containing 10% FBS, 25 µg/ml ascorbate and 50 µg/ml gentamicin]. The discs in the other two groups were first injected with 10 µl of physiologic saline (Saline Group) or OP-1 at 0.2 µg/µl (OP-1 group), using a 28 gauge needle, before being cultured for the next 14 days in the complete medium. This complete medium was replaced daily in all groups.

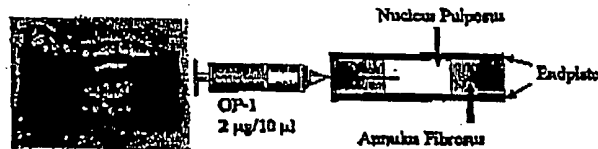


Figure 1 Tissue Culture of IVD with Adjacent End Plates

Biochemical Analyses:

On days 0, 7 or 14 of culture, the endplates were removed and the NP and AF tissues were bluntly separated, lyophilized and weighed. The samples were then digested with papain for 72 hours at 60°C. The digests were analyzed for contents of DNA, using the Hoechst 33258 dye, and for proteoglycan (PG), using the DMB assay [1]. The DNA and PG contents in all groups were normalized to the dry weight.

Statistical Analyses: For each time point, all analyses of discs were performed on 3 sets of one disc each, the standard deviations are shown in the Figure. Statistical analyses were performed using one-way ANOVA with Fisher's PLSD test as a *post hoc* test.

RESULTS:

Evaluation of Tissue Culture System Individual IVDs with an endplate on both sides were readily excisable using a surgical blade. Preliminary culture of the tissue for up to 14 days followed by histological evaluation showed both good retention of matrix in the tissue and maintenance of cell viability.

During the 14 day culture period, no significant change in the dry weight and the DNA content was observed. The PG content of the NT

group decreased significantly between Day 7 and 14 in the NP ($p < 0.05$) but not in the AF.

Effect of Saline and OP-1 Injection The saline injection did not cause any significant change in either the dry weight or the DNA contents of the NP and AF during the culture period. Interestingly, the PG content per unit of dry weight on Day 7 (NP, $p < 0.05$, AF, $p < 0.01$) and Day 14 (AF, $p < 0.01$) was significantly lower in the saline-injected group than in the NT group. As a consequence, further comparisons were limited to examining differences between the saline and OP-1 injection group.

In the OP-1 injection group, the PG content of the NP was significantly higher on Day 14 ($p < 0.01$) than that in the saline group. The PG content in the AF was also higher than that in the saline group on Day 7 ($p < 0.05$) and Day 14 ($p < 0.01$). After 14 days of culture the PG content in NP and AF of the OP-1 injection group was not statistically different from the non-cultured control (Day 0), strongly suggesting that OP-1 contributed positively to the maintenance of steady state metabolism.

DISCUSSION:

In vitro metabolic studies of whole IVDs are considered difficult because the NP tissue swells very rapidly and loses PGs after it is immersed in liquid medium. Although attempts were made to overcome these problems by using one or other modified cultured systems [1, 2], the maintenance of PG steady state metabolism has been extremely difficult to achieve. In our method, the swelling of the NP and the loss of PG in this NP was largely prevented by culturing a whole IVD with its endplates still attached. The results presented here showed that PG contents in the NP could be maintained for 14 days. This method also proved useful in obtaining further support for our contention, based on previous data obtained *in vivo* [3], that a single administration of OP-1 promotes the retention of a high PG content in both the NP and AF. [3].

Finally, this new culture system should enable us to load the IVD tissue in order to investigate the effects of biomechanical stimulation on the metabolism of the extracellular matrix of intact IVDs.

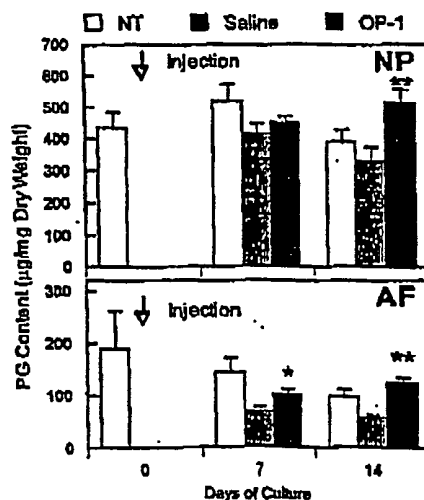


Figure 2. The Effect of OP-1 on the NP and AF in the IVD *in vitro* (* = $p < 0.05$, ** = $p < 0.01$ OP-1 vs. Saline)

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OSTEOGENIC PROTEIN-1 PROMOTES THE FORMATION OF TISSUE-ENGINEERED CARTILAGE USING THE ALGinate-RECOVERED-CHONDROCYTE METHOD

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INTRODUCTION Most attempts to form cartilage *in vitro* use cells cultured within or on a biological or synthetic scaffold. We have developed a novel two-step culture method (alginate-recovered-chondrocyte method, ARC method) for the production of cartilaginous tissue *in vitro* that does not require exogenous matrices [1]. The first step consists of culturing phenotypically stable chondrocytes under conditions optimal for the formation of a cell-associated matrix (CM). The second step allows those cells with their CM to rapidly form and become integrated into a solid mass of cartilage on a porous insert. Cartilage tissue engineered *in vitro* using this approach is softer than normal cartilage: it is rich in proteoglycan (PG), mostly aggrecan and has an immature collagen network. We also have shown that recombinant human osteogenic protein-1 (OP-1) can stimulate PG and collagen synthesis by human and bovine articular chondrocytes. OP-1 promotes both the rate of formation and the size of the CM by these cells. The enhancement of matrix formation in the CM might enable the application of the tissue-engineered tissue to a larger area. For application in humans, the enhancement of the CM formation is preferable, because matrix formation by cells from aged cartilage is limited.

We present here the results of a study aimed at testing the hypothesis that exposure of young adult articular chondrocytes to OP-1 promotes the formation of cartilaginous tissue engineered for transplantation using the ARC method.

MATERIALS AND METHODS The ARC Method was then used as follows to form cartilage *in vitro*.

Step 1: Assessment of Formation of the CM in Alginate Beads Bovine articular chondrocytes from the metacarpophalangeal joints of 14-18 month old steer were isolated by sequential enzyme digestion. Chondrocytes were cultured in beads of 1.2% low viscosity alginate (Keltone LV, Kelco) at 4 million cells/ml using daily changes of medium containing 10% FBS or 10% FBS + OP-1 (100 ng/ml), 25 µg/ml ascorbate and 10 µg/ml gentamicin. At various time points, beads were dissolved by incubation for 20 minutes in 55 mM NaCitate in 150 mM NaCl. The cells with their CM were recovered by mild centrifugation. The contents of sulfated PG and collagen in the CM were measured by the DMB method after papain digestion and RP-HPLC after acid hydrolysis, respectively [2]. The content of hyaluronan (HA) was also assessed using an ELISA [2].

Step 2: Characterization of Cartilage Tissue Formed In Vitro The cells with their CM, recovered after 7 days of culture in alginate, were resuspended in complete medium containing 10% FBS or 10% FBS + OP-1 (100 ng/ml) and seeded onto a tissue culture insert (10mm diameter) with a porous membrane (Costar). After varying times in culture, the *de novo* formed tissue was separated from the porous membrane and the dry weight of the tissue measured. Biochemical analyses were performed as described in Step 1. The tissues were also examined histologically after staining with toluidine blue. For each group, all analyses of tissues were performed on 3 sets of one tissue each. Statistical analyses were performed using one-way ANOVA with Fisher's PLSD test as a *post hoc* test.

RESULTS **Step 1: Formation of the CM in Alginate Beads** On day 7 of culture, the CM formed by chondrocytes cultured in the presence of OP-1 was more voluminous when observed under the microscope. The structure of the cell with its CM was still well preserved after release by citrate buffer. The contents of PG, HA and collagen in the OP-1 group were two to three fold higher than those in the FBS group (Fig 1A). The PG/collagen ratio was higher with OP-1 treatment and decreased with time in culture (Fig 1B).

Step 2: Cartilage Tissue Formed In Vitro Cultured in the presence of OP-1, the individual chondrocytes and their CM progressively became incorporated

into thick, disk-like cartilaginous tissue between 8 and 14 days (step 2: see the histology in Fig 2). The tissue was easy to handle. In contrast, the matrix formed in the presence of 10% FBS alone was highly cellular, but did not form a thick disk-like structure. Histologically, the border of the original CM, formed in the alginate beads culture (step 1) was barely recognized in the *de novo* formed tissue (Fig 2). The dry weight of engineered tissue was significantly more in the tissue with OP-1 (Table). PG, collagen and HA contents in these tissues were significantly higher than those in the tissue formed in the presence of 10% FBS alone. PG/collagen ratio was higher than that of normal articular cartilage but decreased with time in culture.

CONCLUSIONS The results show that OP-1 can significantly enhance the formation of a voluminous tissue *in vitro*. The thickness of the tissue cultured with OP-1 was approximately 4 times that of the tissue cultured with 10% FBS alone (Fig 2). The stimulatory effects of OP-1 on matrix formation were observed in both steps of the ARC method. As the ability of human adult chondrocytes to form a cohesive matrix is limited, the results suggest that human chondrocytes should be stimulated by growth factors, such as OP-1, when the goal is to produce cartilage tissue for transplantation using the ARC method.

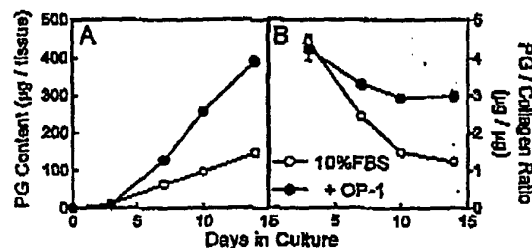


Figure 1. PG Content and PG/Collagen Ratio in the CM (Step 1)

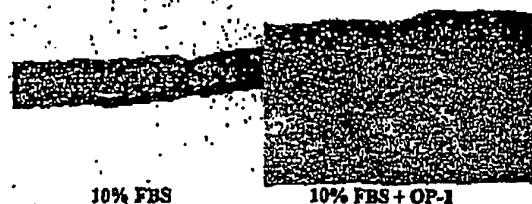


Figure 2: Histology of the Tissue-engineered Tissue

Table	Biochemical Analyses of the Tissue-engineered Tissues	
	10% FBS	10%FBS + OP-1
Dry Weight	1.99 ± 0.08	3.91 ± 0.26 *
PG (µg/tissue)	171.4 ± 9.5	653.6 ± 17.4 *
Collagen (µg/tissue)	120.3 ± 3.8	304.7 ± 9.1 *
PG/Collagen (µg/µg)	1.43 ± 0.11	2.15 ± 0.04 *
HA (ng/tissue)	969.3 ± 34.9	1565.3 ± 87.3 *

(p < 0.01; vs 10%FBS, mean ± SD)

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TRANSFER OF OSTEOGENIC PROTEIN-1 GENE BY GENE GUN SYSTEM PROMOTES MATRIX SYNTHESIS IN BOVINE INTERVERTEBRAL DISC AND ARTICULAR CARTILAGE CELLS

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[INTRODUCTION]

Therapeutic approaches to restore intervertebral disc (IVD) damage in degenerative diseases are limited. Gene therapy is one of the most attractive tool for inducing the expression of growth factors. Non-viral gene delivery system is an alternative that avoids the risk of insertional mutagenesis of retroviruses, immunogenicity of adenoviruses and acquiring replication competence. However direct introduction of genes into the IVD has been consider difficult, in part due to the presence in the tissue of an extracellular matrix composed of dense collagen framework and proteoglycan (PG). We have recently reported the potential of a non-viral gene gun-mediated gene transfer method for efficient transfection [1] of the IVD using a reporter gene.

The purpose of this study was (1) to compare the efficiency of transfection of different IVD [annulus fibrosus (AF) and nucleus pulposus (NP)], and articular cartilage (AC) cells using the gene-gun transfection system and (2) to induce by transfection of the human osteogenic protein-1 (OP-1) gene specific metabolic changes in IVD cells (NP and AF cells) and AC cells.

[MATERIALS AND METHODS]

Cell and Tissue Preparation: AF and NP tissues were isolated from IVD of tails, and AC from the metacarpopharyngeal joints of 14-18 month bovine steers. Cells were isolated by sequential digestion with 0.2% pronase and 0.025% collagenase+0.04% DNase. The cells were seeded at a density of 50,000 cells/well in a 12 well plate and cultured for 2 days prior to transfection. Tissues of IVD and AC were also prepared for gene gun transfection. After the transfection, cells and tissues were cultured in DMEM/F12 medium containing 10% FBS with a daily change of medium.

Reporter Gene and OP-1 Expression Vector: pCMV- β -galactosidase (Clontech) served as a reporter gene, and transgene expression was assessed using the *In Situ* β -galactosidase staining kit (Stratagene). Human OP-1 expression vector, pW24, was kindly gifted by Dr John C. Lee.

Gene Transfer: At the time of gene transfer, a pulse of high pressure helium gas (125 psi) was released from a helium tank through the Gold-Coat tubing, accelerating the DNA-coated gold particles on the inside of the tubing cartridge to penetrate the target cells. The gene gun was positioned at a minimal distance from the petri dish and tissue, and a single bombardment was carried out.

Assessment of Transfection Efficiency: After 3 days, the transfection efficiency of a Lac reporter gene construct (pCMV- β -galactosidase) in the primary monolayer cultures of normal bovine NP, AF, and AC cells was assessed using an *In Situ* β -Galactosidase Staining Kit.

Measurement of Metabolic Activity of Transfected Cells: The DNA content and the total PG content were measured in the cell layer to assess metabolic activity [2]. PG synthesis was also measured using [35 S]-sulfate labeling, followed by rapid filtration [2] and compared between the OP-1-transfected (pW24) and the control (vacant vector) groups. Statistical analyses were performed by one-way ANOVA with Fisher's PLSD test as a *post hoc* test.

[RESULTS]

The gene transfer of β -galactosidase was performed to probe the efficiency of transfection on the three different cell sources. Analysis of X-gal staining demonstrated an efficiency of 10.1% in normal NP cells, 6.2% in AF cells and 5.2% in the AC cells (Figure 1). The DNA content and rate of PG synthesis in none of the three cell types actually differed significantly when comparing the pCMV- β -gal transfected and non-treated groups. This

suggested that the gene gun procedure does not have a significant adverse effect on cell metabolism.

To study whether gene transfection can alter the metabolism of those cells, the human OP-1 gene was transfected using a pW24 vector. On day 3 after transfection, there was no significant difference in the DNA content and PG content of the cell layer in any group. On the other hand, in the OP-1 transfected group, the rate of PG synthesis was significantly higher in all cell types [AC (120%), AF (124%), and NP (144%) cells (Figure 2)]. NP cells were more responsive than AC and AF cells to the transfection of the OP-1 gene.

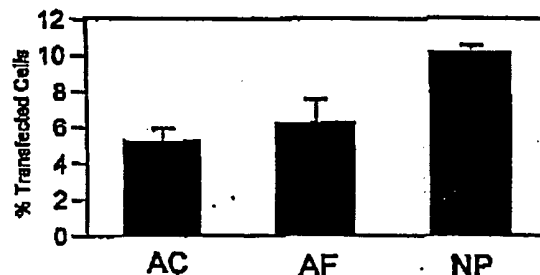


Figure 1. Efficiency of gene gun-mediated transfection in bovine AC, AF and NP monolayer cells.

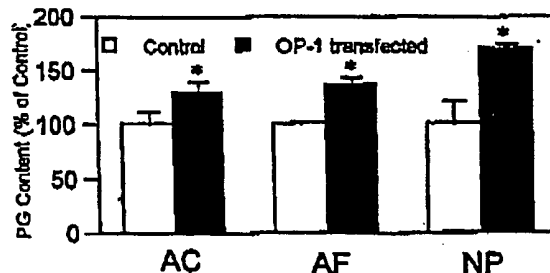


Figure 2. Effect of OP-1 gene transfer on PG synthesis by bovine AC, AF, NP cells. (* $p < 0.05$ versus control)

[DISCUSSION]

The results of this study revealed, for the first time, that transfection of the OP-1 gene by the gene gun system to both IVD and AC cells *in vitro* can alter the metabolism of these cells. Both the efficiency study and the metabolic study provided evidence that the NP cells might be the best target for the transfection.

Although it remains to be proved that OP-1 production was enhanced after gene transfer, this study suggested that gene therapy with the OP-1 expression vector can be a useful method for inducing the regeneration of IVD and AC tissues. Additional studies are now ongoing to determine if the transfection of the OP-1 gene into the IVD and AC tissues can be achieved and if it can influence the metabolism of tissues.

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Category: You have not yet chosen a category.

Poster Only Presentation: No

Author Status: .

Will this abstract be submitted separately to the 2001 ACR Annual Scientific Meeting? Yes.

OSTEOGENIC PROTEIN-1 (OP-1) IN SYNOVIAL FLUID IS A POTENTIAL MARKER IN ARTHRITIS

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Hopkinton, MA.

The development of body fluid biomarkers for arthritis is, potentially, of immense utility in the treatment of these diseases. The molecular analysis of synovial fluid may yield substantially more information than methods routinely used today. The purpose of the current study was to investigate whether osteogenic protein-1 (OP-1) could be detected in synovial fluid, whether quantitative approaches could be adapted for the assessment of OP-1 in synovial fluid, and whether there are differences in the levels of OP-1 comparing normal donors, and patients with rheumatoid arthritis (RA) and osteoarthritis (OA). OP-1 is a member of the bone morphogenetic protein family, and has multiple anabolic effects in many tissues. We have previously demonstrated the expression both of OP-1 protein and OP-1 message in different connective tissues including cartilage, synovium, tendon, ligament and meniscus. For the current study, synovial fluid was aspirated from subjects with OA and RA as well as from normal joints of human organ donors. Specimens from 40 joints were analyzed by Western Blots with anti-pro and anti-mature OP-1 antibodies, and the concentration of OP-1 was measured by a sandwich ELISA method newly developed in our laboratory (Merrihew et al, 2001),* which was tested and optimized for the analysis of synovial fluid. We found pro and mature forms of OP-1 simultaneously present in all tested samples. The distribution of immunoreactive bands was similar to that described for human articular cartilage. By ELISA, clear differences in OP-1 levels were identified between OA and RA groups. Notably, the content of OP-1 in synovial fluid from organ donors was comparable to that detected in cartilage extracts from the same donors. OP-1 concentration was higher in donors with normal knee joints than in donors with degenerative changes ($p < 0.01$). Additionally, OP-1 concentration was higher in synovial fluid obtained from RA patients than in that from OA patients ($p < 0.04$). The results of this study suggest potential for development of synovial fluid OP-1 as a diagnostic and prognostic marker.

* as disclosed herein previously

I claim:

1. A method of diagnosing inflammatory joint disease, said method comprising the step of determining the concentration of detectable OP-1 in a joint, wherein said OP-1 concentration is an indicia of inflammatory disease.
2. The method of claim 1 wherein said concentration of detectable OP-1 is measured by using ELISA methodology.
3. A method of monitoring skeletal tissue diseases, said method comprising the step of determining the concentration of detectable OP-1 in a skeletal tissue, wherein said OP-1 concentration is an indicia of skeletal tissue disease.
4. The use of OP-1 as a body fluid biomarker for diagnosis and prognosis of inflammatory joint disease.
5. The body fluid of claim 4 wherein said fluid is synovial fluid.
6. A method of monitoring age-related tissue changes, said method comprising the step of monitoring the concentration of endogenous OP-1 as a function of time.
7. The skeletal tissue of claim 3, wherein said tissue is selected from the group consisting of: cartilage, ligament, meniscus, tendon, and synovium.
8. The skeletal tissue of claim 3, wherein said tissue is synovial fluid.

CHEMILUMINESCENT SANDWICH ELISA

1. Coat plate with 50ng/well, anti OP-1 antibody (Santa Cruz # SC- 9305) in TBS, pH 7.5 and incubate at 4°C overnight.
2. Wash wells 4 times with TBS/T (0.1% Tween 20 in TBS, pH 7.5).
3. Dispense 200µl of Blocking solution (5% non fat dry milk, BIORAD # 170-6404 in TBS/T, pH 7.5) into each well and incubate plate at room temperature (RT) for 2 hours.
4. Wash wells 4 times with TBS/T.
5. Prepare standard mature OP-1 dilutions in TBS/T ranging from 10ng/ml to 0.01 ng/ml.
6. Add 100 µl of diluted standard, sample (extract) in each well and incubate plate at RT for 1 hour.
7. Wash plate 4 times with TBS/T.
8. Add 100 ul/well of diluted 1B12 antibody (1:1000) in TBS/T and incubate plate at RT for 1 hour.
9. Wash plate 4 times with TBS/T.
10. Add 100 µl/well of diluted (1:10,000) ImmunoPure Goat Anti-mouse IgG (H+L) (min x) Peroxidase Conjugated (PIERCE # 31434) in TBS/T and incubate plate at RT for 1 hour.
11. Wash plate 4 times with TBS/T.
12. Add 100 µl/well of Supersignal ELISA Femto Maximum Sensitivity Substrate (PIERCE # 37075) (prepared by mixing equal parts of Supersignal ELISA Femto Luminol/Enhancer solution and Supersignal ELISA Femto Stable peroxide solution).
13. Mix liquid in wells for 1 minute on a shaker.
14. Read the Relative Light Units (RLU) with a luminometer.

OP-1 Sandwich ELISA

Day 1

Label white, detachable chemilluminescent plates, #1-12

Record template of samples to be tested

Coat plates: Santa Cruz Antibody 100 ng/well, 50 ul/well

Ex (96 wells): 25 ul SC/5 ml TBS, pH 7.5

Tap plate to cover bottom of wells, check for bubbles

Cover plate with plate sealer, 4°C ON

Day 2

Prepare 2 L wash buffer: 2L TBS, pH 7.5/2.2g Tween 20

Prepare 5% milk in wash buffer

Ex (96 wells): 1g milk, 20 ml wash buffer

Wash plate 3X's with wash buffer, 200ul/well

Block: 5% milk 200 ul/well

Cover plate, RT, 2 hrs

Prepare OP-1 standard dilutions:

Prepare OP-1 standard dilution											OP-1
<div>1ul 50ul 100ul 500ul 500ul 500ul 500ul 500ul 500ul 500ul 100ul</div>											Tube #
STOCK	A	1	2	3	4	5	6	7	8	10	
1mg/ml OP-1											Wash Buffer
	500ul	950ul	900ul	500ul	500ul	750ul	500ul	500ul	750ul	500ul	STD CONC
		100ng	10ng	5ng	2.5ng	1ng	0.5ng	0.25ng	0.1ng	0.05ng	
										0.01ng	

Wash plate 4X

Add standards or samples: 100ul/well, in triplicate

For blanks (run on each plate used) add 100ul wash buffer

Cover plate, RT, 1 hr

Wash plate 4X

Add sandwich antibody: 1B12, 1:1,000, 100ul/well

Ex (96 wells): 10ul 1B12, 10 ml wash buffer

Check for bubbles

Cover plate, RT, 1 hr

Wash plate 4X

Add secondary antibody: Pierce anti-mouse HRP, 1:10,000, 100ul/well

Ex (96 wells): 1ul Anti-mouse Ab, 10 ml wash buffer

Cover plate, RT, 1 hr

Wash plate 4X

Substrate: Super Signal ELISA Femto Chemill Substrate, mix 1:1, 100ul/well

Ex (96 wells): 5 ml A, 5 ml B

Wipe pipet tips with paper towel in between rows

Check for bubbles

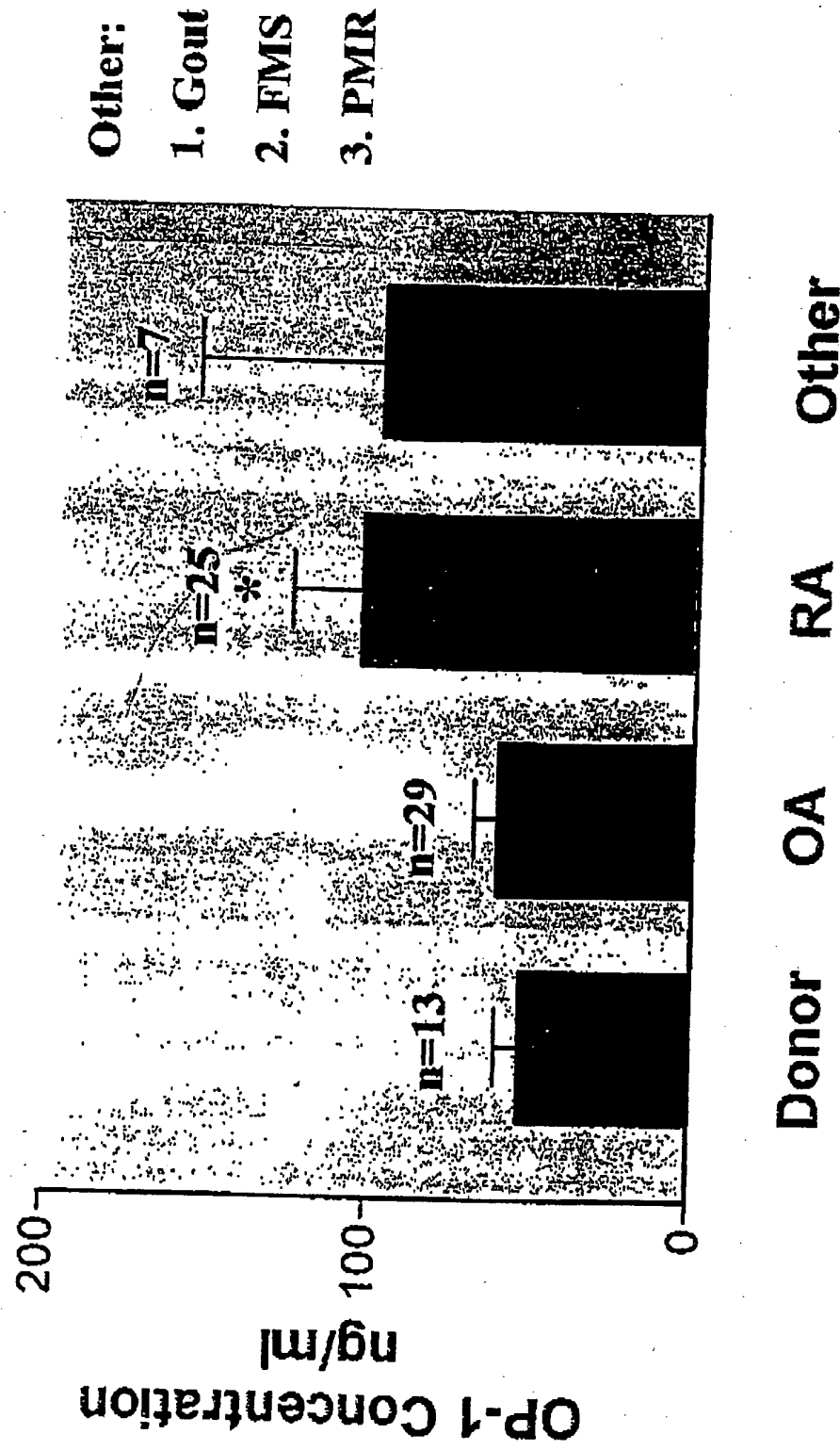
Cover and rotate for 1 min

Get RLU counts

PROTOCOL FOR EXTRACTION OF PROTEIN FROM CARTILAGE

- Weigh cartilage tissue samples (0.5 gm), lyophilize overnight and weigh the tissue again to get dry weight of tissue.
- Pulverize samples in liquid nitrogen.
- Add 1 protease inhibitor tablet (Roche Diagnostics # 1836153, complete mini protease inhibitor tablets) per 10 ml of ice cold 1M GuHCl buffer (1M GuHCl, 10mM CaCl₂ & 50 mM Tris, pH 7.5). Add 3.5 ml buffer per 0.15 gm (dry weight) of tissue.
- Incubate at 4°C, with rotation for 4 hours.
- Transfer supernatant to microcentrifuge tubes and centrifuge at 2500 rpm for 10 minutes at 4° C. Collect supernatant and store at 4° C.
- Dialyze for 2 days against water (12,000-14,000 MW cut off).
- Test extracts by OP-1 chemiluminiscent sandwich ELISA.

Content of endogenous OP-1 in human synovial fluid



$P < 0.15$ Donor vs RA; $P < 0.03$ OA vs RA

FMS = Fibromyalgia Syndrome

PMR = Polymyalgia Rheumatica

OP-1 Sandwich Chemiluminescent ELISA

Coat Antibody: SC
polyclonal OP-1

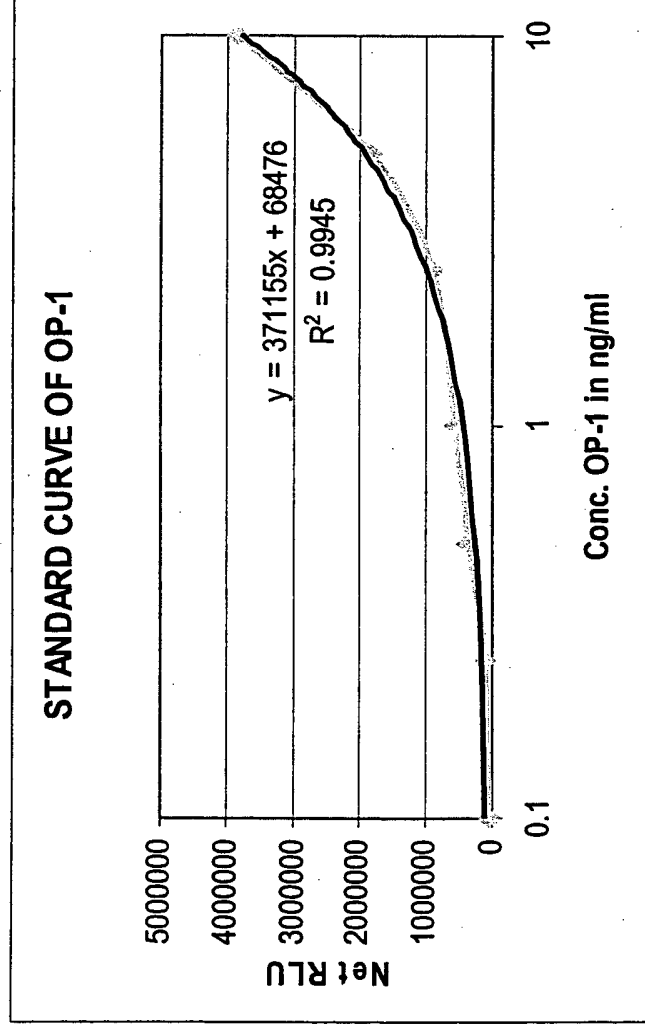
Std Curve: mOP-1

Second ab: 1B12
monoclonal OP-1

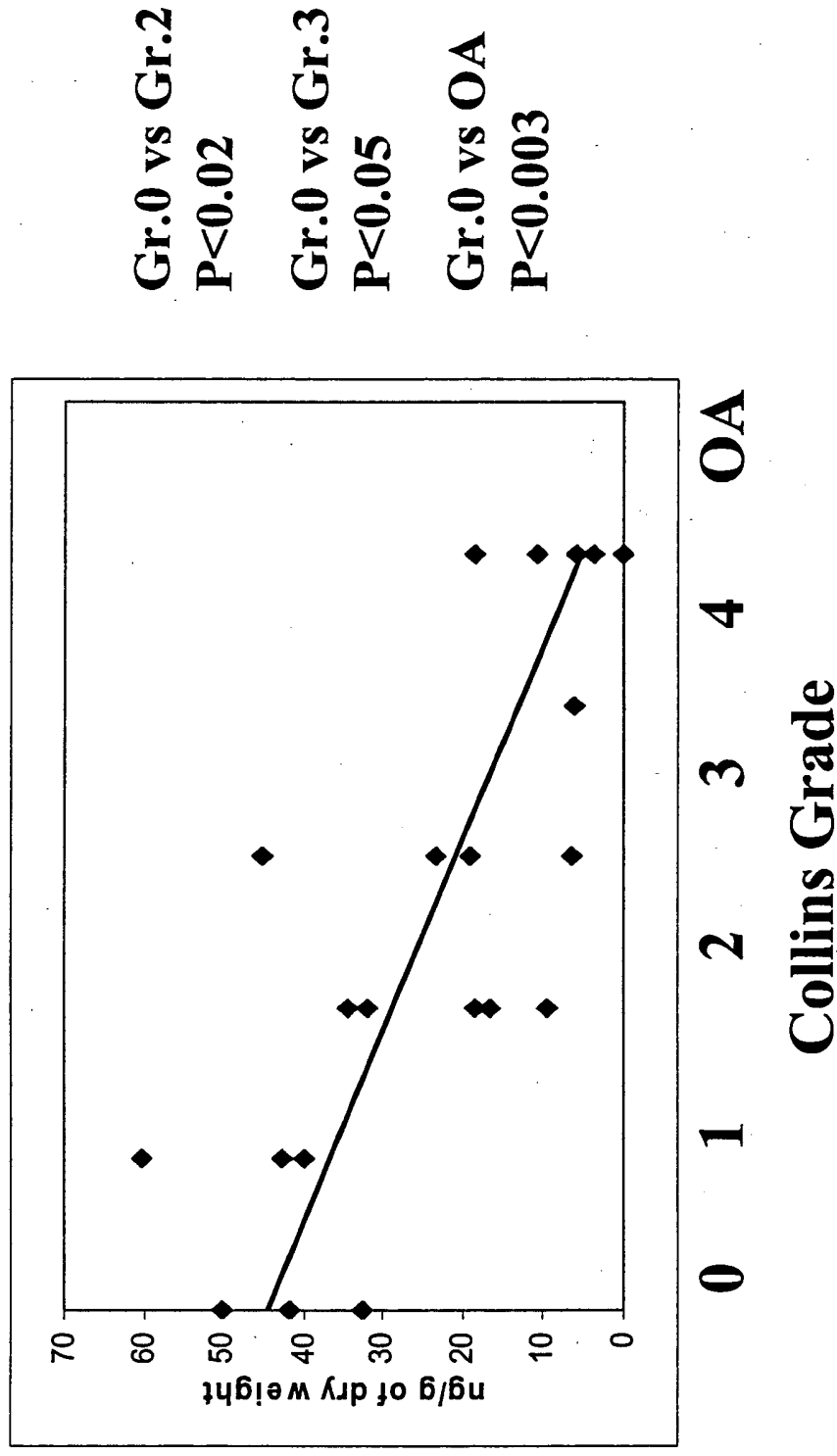
Third Ab: Goat Anti
Mouse HRPO
(Pierce)

Substrate: *Femto*
Chemiluminescent
Substrate (Pierce)

pH 7.5

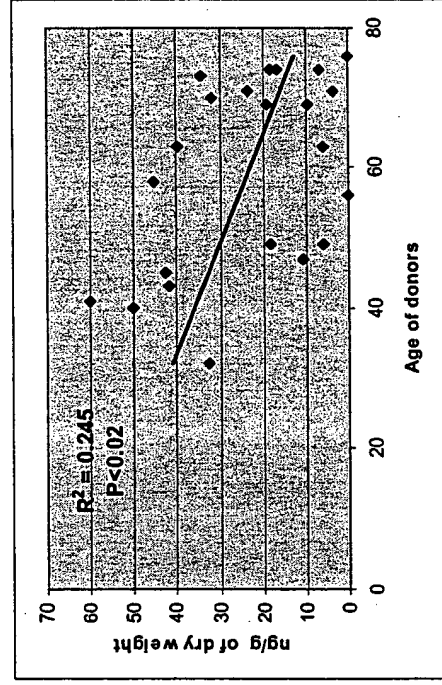


Content of endogenous OP-1 detected by ELISA in extracts from normal and OA knee cartilage

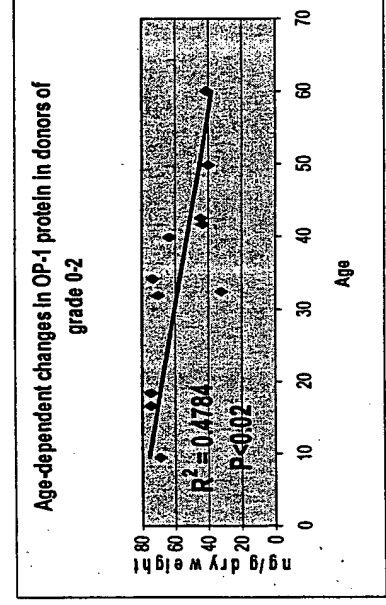
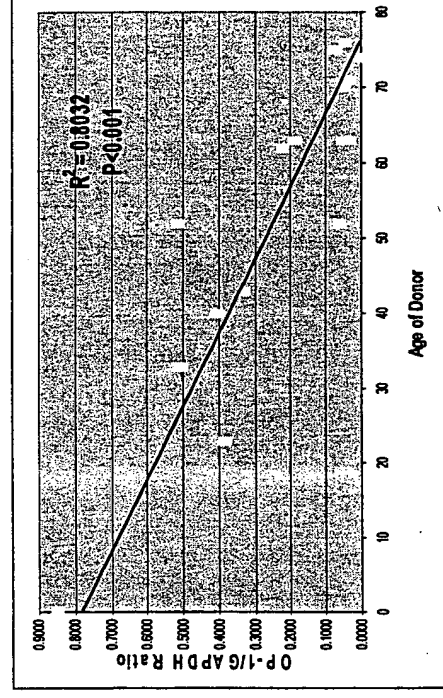


Age-related changes in endogenous OP-1

ELISA

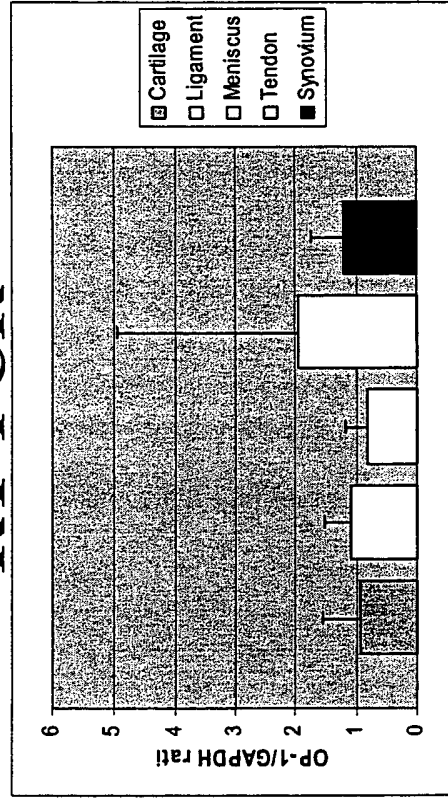


RT-PCR

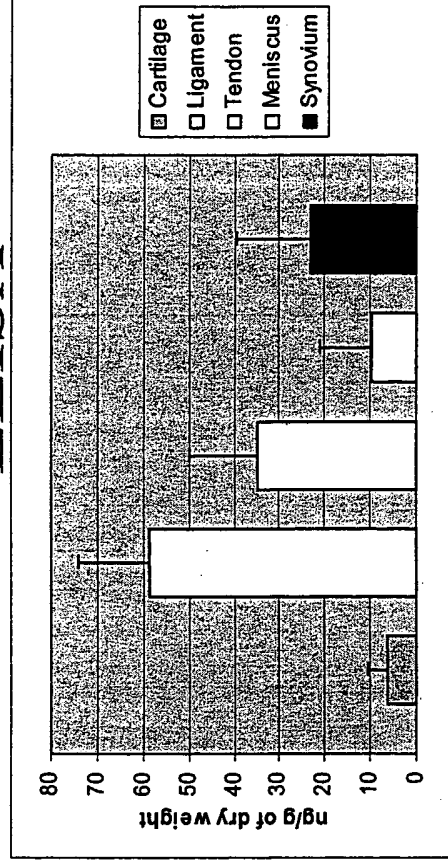


Endogenous OP-1 in connective tissues of the knee joint (N=3)

RT-PCR

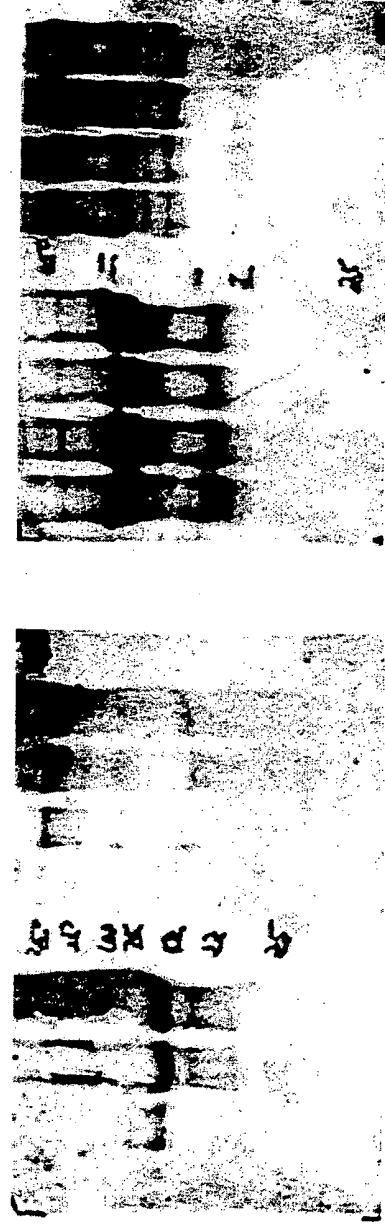


ELISA



Western blotting of synovial fluid with anti-mature OP-1 antibody (R&D)

Reduced Non-Reduced Reduced Non-Reduced



1 - 1:1000

2 - 1:100

3 - 1:10

4 - 1:5

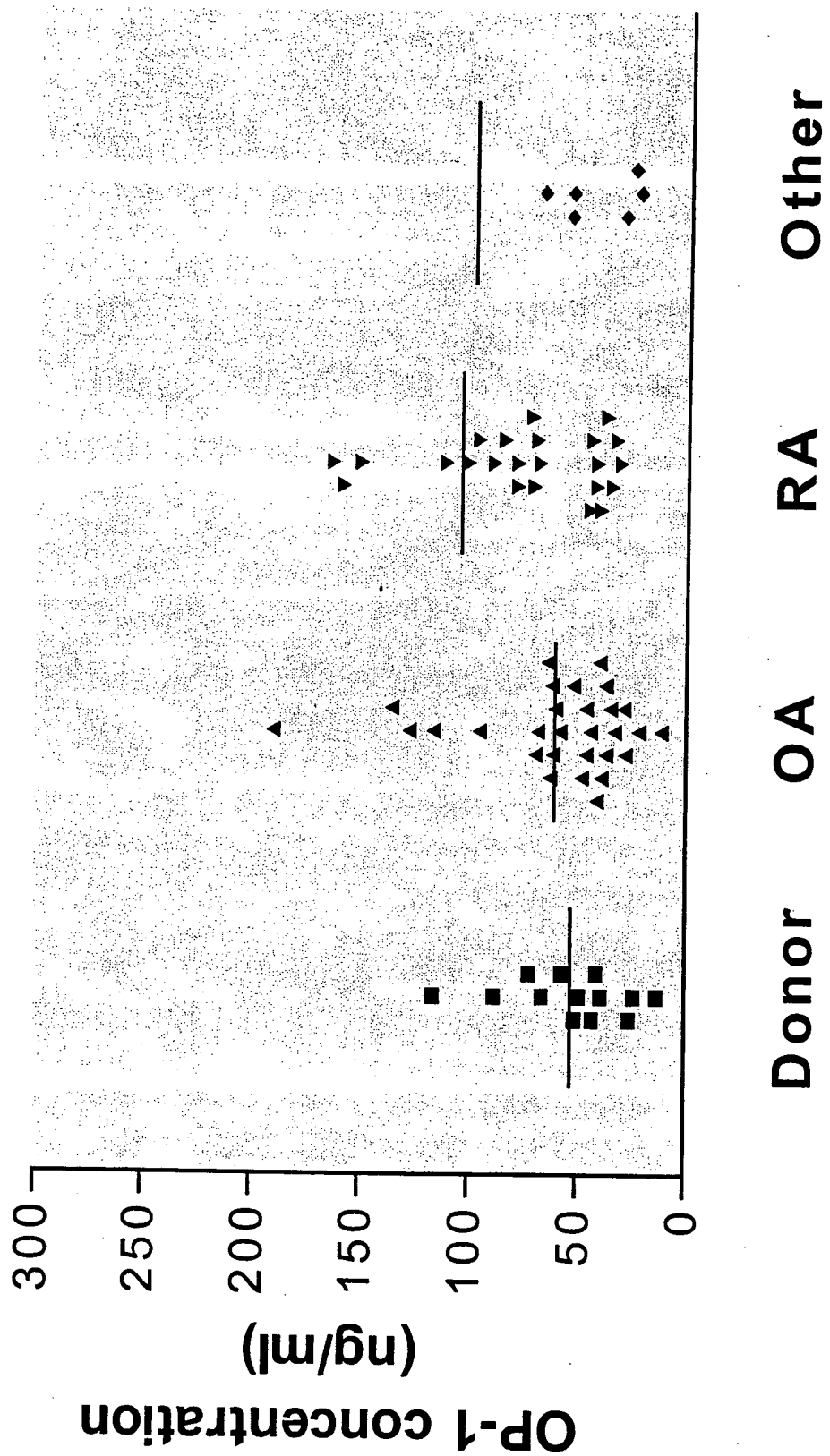
a - no treatment

b - hyaluronidase

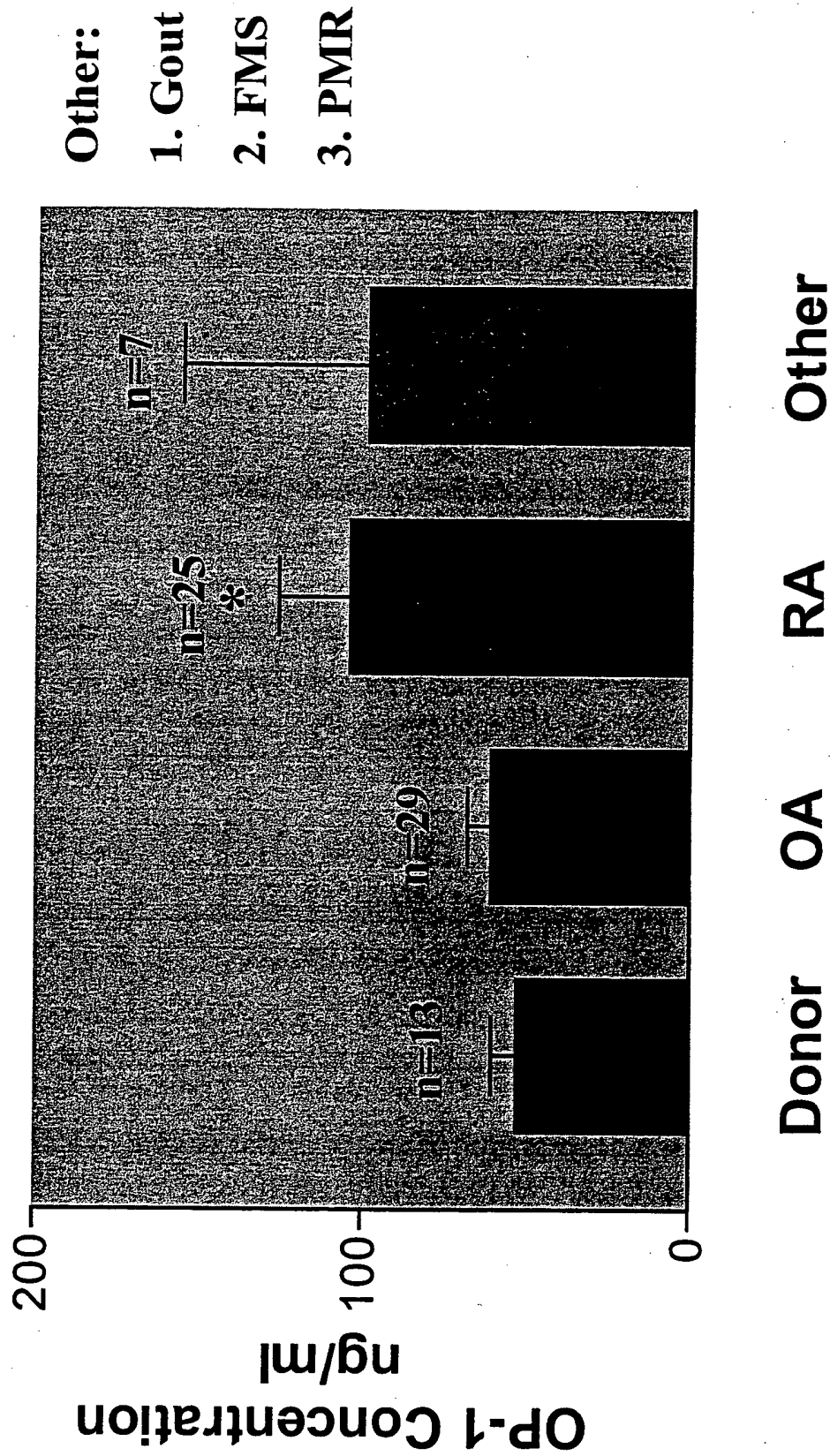
c - chondroitinase

d - b+c

Content of endogenous OP-1 in human synovial fluid

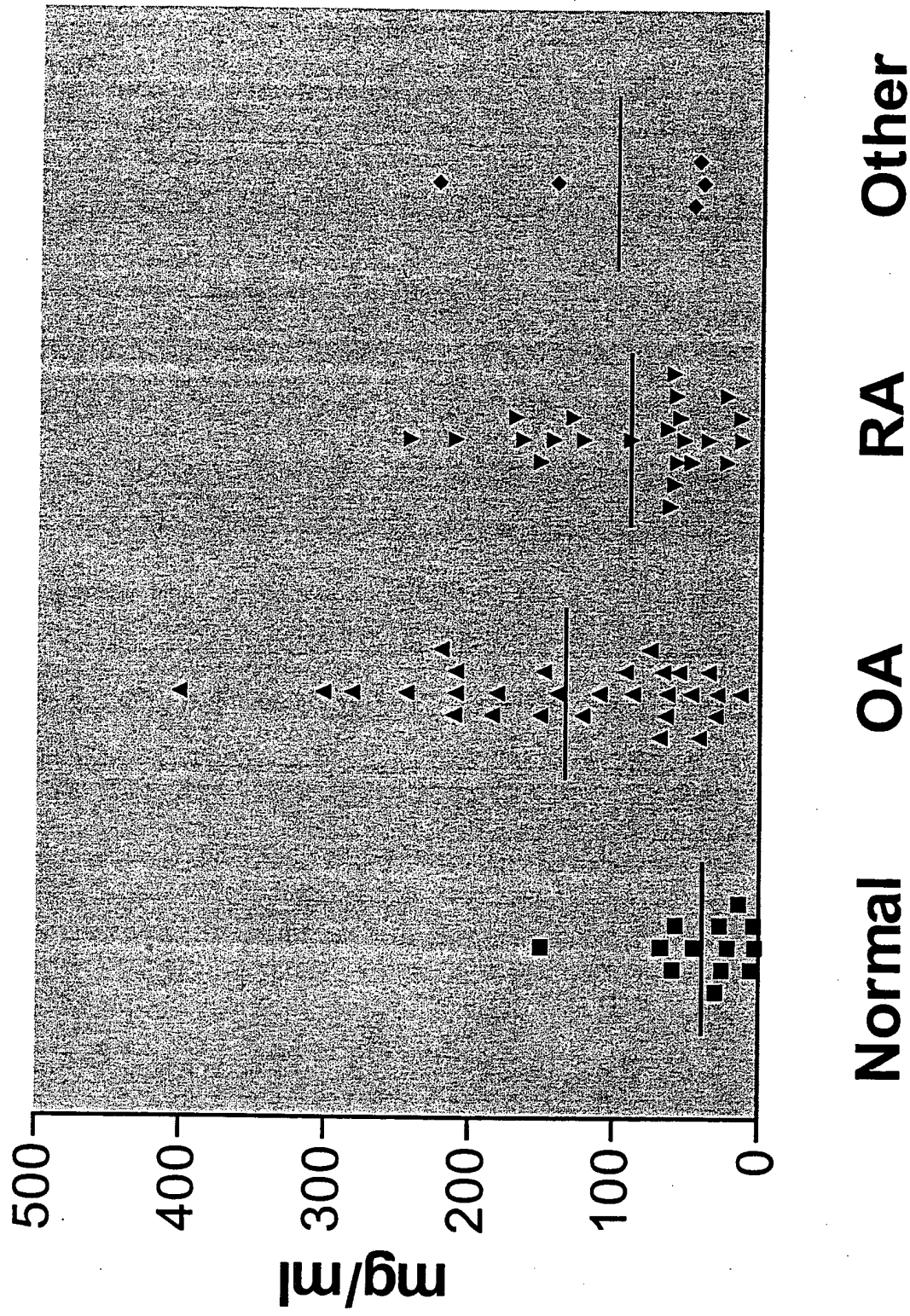


Content of endogenous OP-1 in human synovial fluid

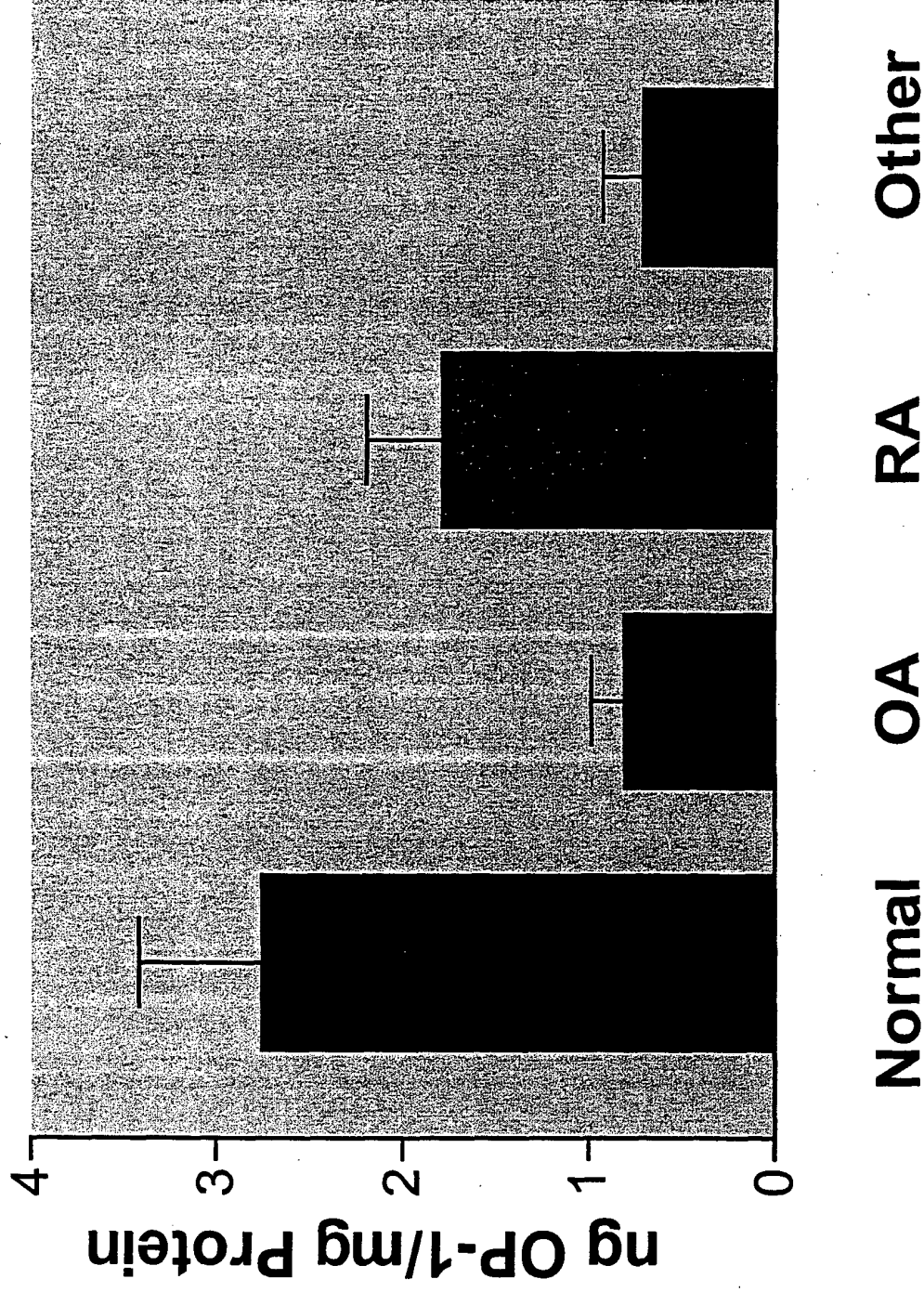


*P<0.15 Donor vs RA; P<0.03 OA vs RA

Total Protein Concentration in Synovial Fluid



Content of Endogenous OP-1 in Synovial Fluid Normalized to Total Protein



Conclusions

- Endogenous OP-1 could be detected in synovial fluid from normal donors, OA and RA patients.

Concentration of endogenous OP-1 is two-fold up-regulated in synovial fluid of the RA group as compared to OA and normal groups.

When normalized to total protein, concentration of OP-1 is still two-fold higher in synovial fluid from RA patients compared to values detected for OA patients.

- No correlation between changes in total protein and OP-1 content has been detected which might suggest that these two parameters are independent phenomena.